SYMBIOSIS OF HYDRA AND ALGAE.
II. EFFECTS OF LIMITED FOOD AND STARVATION ON GROWTH OF SYMBIOTIC AND APOSYMBIOTIC HYDRA

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Very few measurements have been made on the effect of symbiotic algae on growth of their various invertebrate hosts. Recently Karakashian (1963) demonstrated that the algae symbiotic with *Paramecium bursaria* exert a strong influence on the growth of the host. These studies were carried out with symbiotic and aposymbiotic individuals of known genetic and nutritional history cultured in a defined medium. Culture techniques (Loomis, 1954; Muscatine and Lenhoff, 1965) now permit a similar approach using green hydra, thus affording insight into an association of algae and a metazoan. Previous studies on the role of algae in green hydra (Goetsch, 1924; Haffner, 1925) were carried out in undefined media, and lacked the quantitative precision necessary for critical evaluation.

The present study describes experiments on the growth, survival, and protein turnover of hydra with and without algae as a function of exogenous food supply. Possible mechanisms of interaction between algae and host are discussed. A preliminary note on some of this work has appeared elsewhere (Muscatine, 1961).

Materials and Methods

All experiments were carried out with *Chlorohydra viridissima*, Carolina strain 1960.The culture medium, and methods for maintaining animals in the laboratory, sampling individuals for experiments, obtaining algae-free controls and conducting growth experiments are described in a previous paper (Muscatine and Lenhoff, 1965).

"Pale green" hydra containing known amounts of algae intermediate between green and albino (= algae-free) were obtained in the following manner. Green hydra were placed in culture solution containing 0.068 *M* glycerine, which causes the gradual elimination of algae (Whitney, 1907, 1908). At daily intervals for eight days, groups of ten "uniform" (*cf.* Lenhoff and Bovaird, 1961) hydra were removed, rinsed in clean culture solution, and exposed to $C^{14}O_2$ for exactly 24 hours, using a procedure described by Muscatine and Lenhoff (1963). These labeled animals were then rinsed in several changes of clean culture solution, and placed on a Millipore filter (HA-47) in a drop of deionized water. When relaxed, the animals were flattened on the filter by application of suction (*cf.* Lenhoff, 1959).

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The filter was dried, glued to an aluminum planchet and assayed for radioactivity. The level of radioactivity of untreated green hydra controls was considered to represent the net photosynthetic activity of the normal algal flora. Glycerine-treated animals having fewer algae had proportionally less radioactivity. Albinos served as controls for animal fixation of $C^{14}O_2$. Figure 1 shows the radioactivity of each group plotted against time grown in glycerinated culture solution. Hydra sampled after four and six days of glycerine treatment were judged to contain,

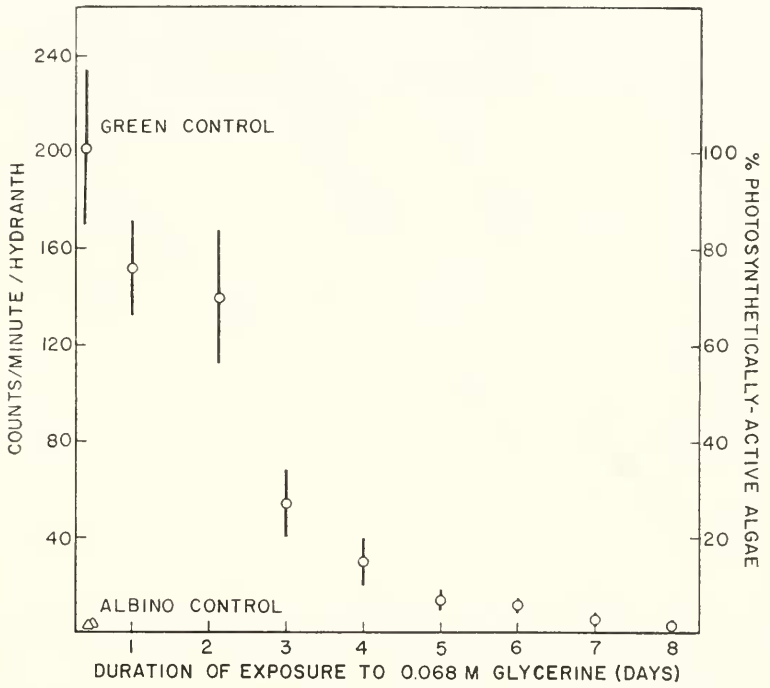

FIGURE 1. Radioactivity accumulated by green hydra exposed first to 0.068 *M* glycerine for periods up to 8 days, and then to $C^{14}O_2$ for 24 hours. Vertical bars denote twice the standard deviation of the mean number of counts per minute. Non-glycerine-treated green controls, in 18 trials, gave 203.2 ±32.0 counts per minute per hydranth. Albinos gave less than two counts per minute above background.

from this reference curve, approximately 10–20% and 4–6%, respectively, of their usual normal complement of algae. These animals were washed with several changes of clean culture solution one hour before experiments.

To graft the heads (hypostome and tentacles) of green hydra onto the bodies (gastric region and below) of albinos, one-day starved stock hydra were bisected transversely. Appropriate pieces were threaded on a hair and held together by gentle pressure with watchmaker's forceps. Adhesion began within a minute or two and grafts were available after 15–30 minutes. The approximate algal content of green heads was estimated by first exposing whole intact "uniform" green hydra to $C^{14}O_2$ in a standard manner (Muscatine and Lenhoff, 1963), and

then cutting each animal in two just below the hypostome and tentacles. Each head and body was then dried separately on a planchet and assayed for radioactivity. In five replicates, green heads were found to contain $30.5 \pm 2.3\%$ of the normal complement of photosynthetically-active algae in an entire animal.

S^{35}-labeled mouse liver (specific activity 1000–2500 counts per minute per microgram protein nitrogen) was prepared, administered to hydra and fractionated as described by Lenhoff (1961). Radioactive material was assayed, with correction for background, with an end window gas flow counter (Nuclear-Chicago C111-B).

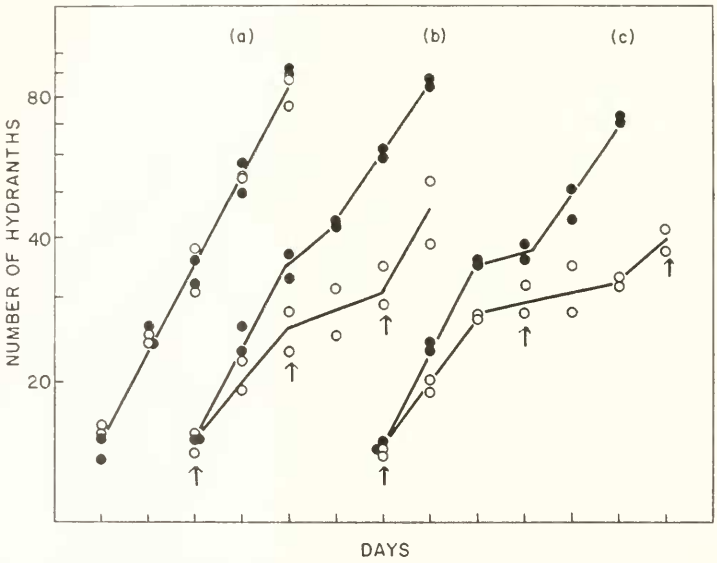

FIGURE 2. Semi-logarithmic plot of growth of green (closed circles) and albino (open circles) *C. viridissima* (a) fed daily, (b) fed every second day, and (c) fed every third day. Arrows indicate time of feeding.

RESULTS

1. *The effect of amount of food on growth of green and albino C. viridissima*

In a previous paper (Muscatine and Lenhoff, 1965) we reported that green and albino *C. viridissima* grew at nearly identical logarithmic rates when fed daily on excess *Artemia* nauplii. This is illustrated in Figure 2, curve a. A doubling time of about 1.5 days is the maximum growth rate (k_{max}) for this species under these conditions (Muscatine and Lenhoff, 1965). Growth of albinos at k_{max} indicates that algae are not essential for logarithmic growth as long as there is ample exogenous food. However, when food was limited, growth rates of green hydra always exceeded those of algae-free individuals, as shown in curves b and c. Curve b shows that the growth rate of green hydra fed every second day deviated only slightly from the rate of animals fed daily. Growth of albinos, on the other hand, lagged after the second feeding, and increased only after a third feeding. Green hydra produced nearly twice the

number of buds produced by albinos. When the diet of excess *Artemia* nauplii was further limited to a feeding every third day (curve c), growth of green hydra dropped off sharply during the first two-day interval without food, but resumed a nearly normal rate immediately after the next feeding. Growth of albinos also dropped off after two days without food but continued to lag through the second feeding without resuming a normal rate. Again, green hydra produced almost twice the number of buds produced by albinos.

Since hydra are normally given excess *Artemia* larvae at a feeding, there was the possibility that in experiments with limited feeding, green hydra had simply taken in more food. This was tested by feeding green and albino hydra daily with single *Artemia* nauplii. This regime both controlled and limited the food intake. Freshly hatched larvae were fed to individual green and albino hydra with a tapered pipette allowing the larvae to leave singly. Since the number of

TABLE I

Growth of replicate cultures of green (G) and albino (A) C. viridissima fed daily but only on single Artemia nauplii. Numbers in parentheses indicate total number of shrimp given to each culture

		No. of hydranths on day							
Exp.		1	2	3	4	5	6	7	k
			(5) (9)	(15)	(19)	(23)	(30)		
1	G	10	17	22	30	41	60	71	0.277
	G	10	18	25	31	41	57	71	0.277
	A	10	20	23	25	30	35	36	0.121
	A	10	17	19	22	27	37	39	0.187
			(2) (3)	(3)	(4)	(5)	(8)		
2	G	4	7	8	10	13	21	—	0.346
	G	4	5	8	9	11	17	—	0.277
	G	4	6	8	9	11	17	—	0.277
	A	4	5	6	6	9	11	—	0.198
	A	4	6	6	7	7	12	—	0.210
	A	4	5	6	7	10	12	—	0.231

hydranths in each culture changed as the experiment progressed, a second shrimp was given to some individuals in order that cultures would receive the same number of shrimp. In this case the additional larvae were fed to maturing buds. Table I shows that under these conditions the average growth rate of green hydra (0.29) still approached that of well-fed individuals, while the average for albinos (0.19) was significantly lower ($p < 0.05$).

Some experiments were carried out to determine if a greater capacity for gastrodermal phagocytosis might have accounted for the increased growth of green hydra on a limited food supply. Twelve green and 12 albino hydra were each fed a small piece of S^{35}-labeled mouse liver along with excess *Artemia* nauplii. Fractionation by differential solubilities showed that 80% of the isotope was bound in the alcohol, trichloroacetic acid-insoluble liver fraction, *i.e.*, the residual protein fraction, and was thus favorable for tracing the course of food protein from the gut lumen into phogacytic digestive cells. At hourly intervals during the six

hours following ingestion, duplicate pairs of green and albino hydra were bisected longitudinally, and the gut contents (ingested but not phagocytized) were washed out with culture solution onto a planchet. The radioactivity of this material was then measured and compared to that remaining in the hydra tissues (phago-

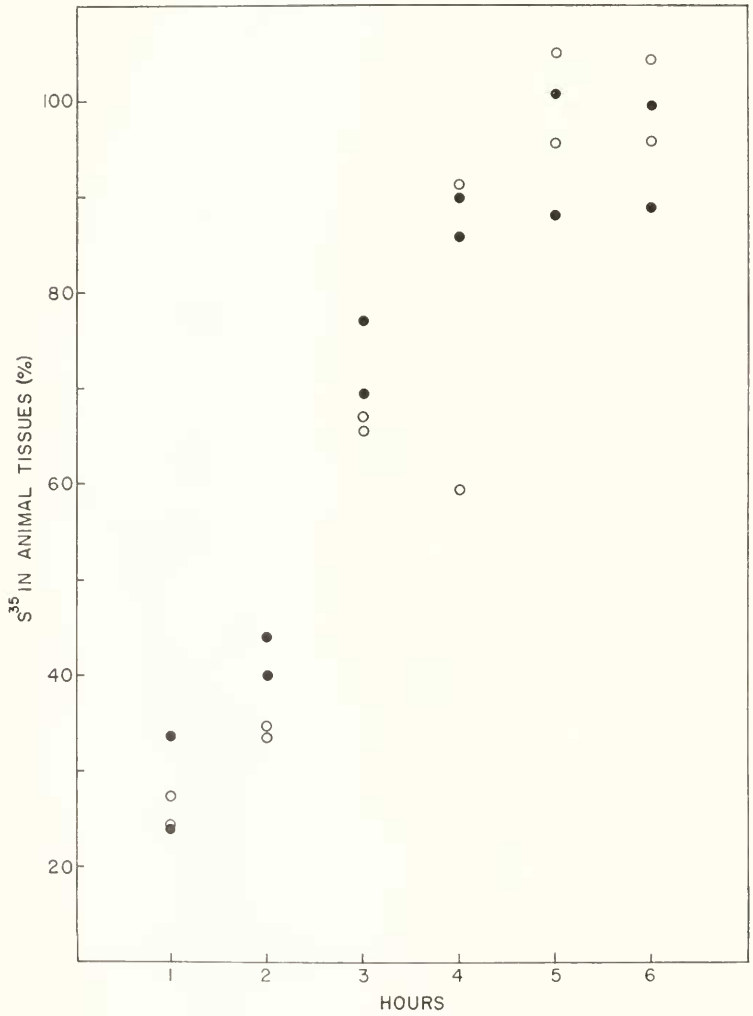

FIGURE 3. Rate of phagocytosis of S^{35}-labeled mouse liver by replicate cultures of green (closed circles) and albino (open circles) *C. viridissima*.

cytized). The curve in Figure 3 represents the rate of phagocytosis of sulfur-labeled tissue. Phagocytosis proceeded relatively slowly over the first two hours, more rapidly during the next two to three hours, and then more slowly after five to six hours as the phagocytic capacity of gastrodermis reached a maximum. Both green and albino hydra phagocytized 85–95% of the labeled tissue and at similar

rates, indicating that the absence of algae did not impair the phagocytic capacity of albino *C. viridissima*. Thus, the difference in growth of green and albino on a limited food supply was not simply the result of a quantitative difference in food intake. This conclusion is further borne out by starvation experiments.

2. *The effect of starvation on survival of green and albino C. viridissima*

Goetsch (1924) described an experiment in which green and albino hydra were placed in the same aquarium with little food and the albinos gradually died out. He concluded that albinos live only when well-supplied with food. These observations were confirmed by randomly placing 10 green and 10 albino *C. viridissima* in a 10-gallon aquarium containing aged tap water, several *Gambusia* sp., common aquatic plants, and a sparse population of an unidentified ostracod. This laboratory "ecosystem" was observed daily but otherwise unattended. After three weeks the number of green hydra had at least trebled while no albinos could be found.

TABLE II

Results of 8 replicate experiments showing the mean (± standard deviation of the mean) number of green, pale green and albino C. viridissima surviving starvation, and the range of survival times

Group	% algae	No. of hydranths on day								Range of survival (days)
		0	2	4	6	8	10	12	14	
Green	100	10	20.5±1.5	24.7±1.5	28.5±1.9	29.5±4.3	29.0±2.4	32.5±0.7	31.0±0.0	28–30
Pale green	10–20	10	20.7±1.7	24.2±3.1	23.7±3.4	23.4±3.7	23.0±3.6	21.0±4.9	22.2±3.2	24–26
Pale green	4–6	10	21.0±1.0	25.5±3.5	24.5±3.5	24.5±3.5	21.0±6.0	13.0±4.0	8.0±6.0	17–20
Albino	0	10	18.2±3.5	20.6±3.7	18.7±4.3	12.5±4.2	5.6±3.2	1.7 —	0.6 —	10–12

To obtain quantitative data on starvation, five green hydra were placed in 30 ml. of culture solution in a Petri dish (100 mm. × 15 mm.). Five albinos were similarly treated. Also starved in the same manner were two different groups of five "pale green" *C. viridissima,* one containing 4–6% and the other 10–20% of the normal algal flora. The animals were illuminated but not fed, and the culture medium was changed once daily. The number of hydranths in each vessel was recorded daily. The results are shown in Table II. During starvation green hydra produced buds for 12 days and survived for nearly four weeks, gradually becoming smaller during this time, and finally disintegrating. "Pale green" individuals did not appear to change, judging from their relative shades of green, until after about 10 days of starvation when the 5% "pale green" group seemed noticeably whiter. Albinos produced buds for about 6 days and most survived for only 10–12 days. One or two individuals, in subsequent starvation experiments, survived for as long as 17 days. Unlike green and pale green hydra, most of the albinos disintegrated soon after they discontinued budding. This unusually premature event was characterized by crumbling of tentacles and body tube until all that remained of each albino was an amorphous accumulation of whitish debris. It was frequently difficult to decide when an albino was "dead." This was arbitrarily taken as the time at which the crumbling of

tentacles was first noticed. Goetsch also observed the disintegration of starving albinos in contrast to the gradual diminution of similarly treated green hydra.

From the data on starvation in Table II, it was possible to estimate the degree to which the number of algae influenced survival. In Figure 4 the percentage of algae contained initially by the hydra in each group is plotted against (1) the average number of hydranths present at 12 days starvation, and (2) the range of maximum survival times. In the first curve, 12 days was chosen because it represents the time at which no albinos remain, although data from 8–14 days give curves of essentially the same character. The shape of the resulting curves is interpreted to mean that survival ability of a starved *C. viridissima* is not appreciably impaired

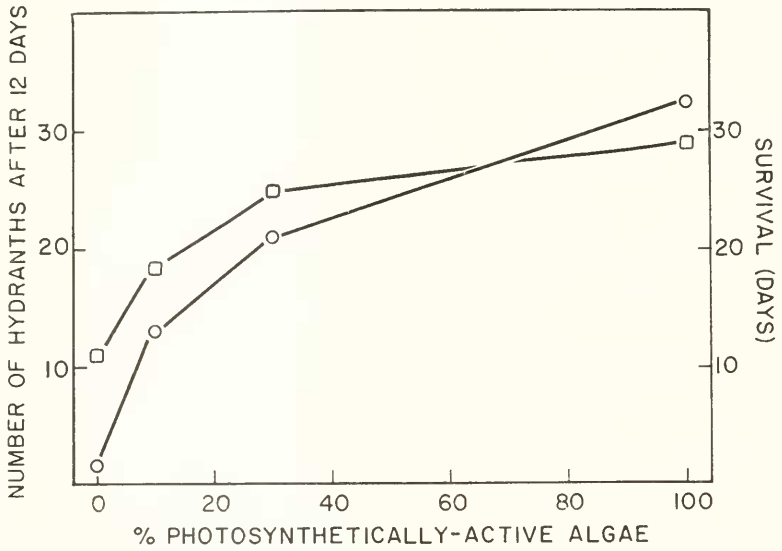

FIGURE 4. Survival of starved *C. viridissima* as a function of the number of algae contained. Using data from Table III, the per cent of photosynthetically-active plant material is plotted against the number of hydranths present after 12 days' starvation (open circles) and mean survival (open squares).

until the level of its photosynthetically-active plant material drops below 15–20% of its normal value. Thus, only about 5–10% of the normal algal flora appears necessary for half-maximum survival ability of the starving host.

3. *The effect of starvation on turnover of S^{35}-labeled food*

To compare the turnover of protein by starving green and albino *C. viridissima*, we measured the rate at which radioactivity was released into the medium by hydra which had previously ingested S^{35}-labeled mouse liver. Duplicate groups of 10 green, 20 albino, and 10 "pale green" (15% algae) hydra were fed sulfur-labeled liver and allowed to regurgitate the uneaten portion 6 hours later. Immediately after regurgitation 10 of the labeled albinos were decapitated and unlabeled green heads of known algal content were grafted onto the labeled albino bodies as

described under Methods. Each group of hydra (green, "pale green," albino, and graft) was placed in 2 ml. of culture solution in depressions of plastic temperature control blocks (Coral Research and Development, Miami, Fla.) maintained at 22.5 ±0.25° C. At 24-hour intervals for five days, the culture fluid was removed from each group and the animals and vessels were rinsed with 0.5 ml. of culture solution per group. The solution and rinsings were combined, dried on planchets and assayed for radioactivity. A fresh 2-ml. portion of culture solution was added to the hydra. After five days the animals were removed and assayed for radioactivity. The sum of the radioactivity of fluid samples is the total S^{35} present at the beginning of the experiment. Material released is expressed as a per cent of this total. In five experiments (Table III) the loss of S^{35} by the groups of hydra always bore the same relationship, although considerable variation was encountered from one experiment to another. Figure 5 shows the rates of loss in one

TABLE III

Per cent of S^{35} lost by groups of green, albino, pale green (10–20% algae) and grafted (30.5% algae) C. viridissima during 5 days' starvation

Expt.		Green	Albino	Pale	Graft
1	a	10.8	22.8	—	—
	b	14.2	23.5	—	—
2	a	19.9	40.5	—	—
	b	23.2	33.2	—	—
3	a	19.1	89.5	29.6	28.2
	b	29.9	53.5	24.4	31.0
4	a	12.0	24.6	—	—
	b	14.0	34.8	—	—
	c	11.1	35.4	—	—
5	a	35.2	68.5	57.3	48.1
	b	40.6	82.1	46.3	61.1

experiment. Invariably albinos lost material to the medium faster than any other group. Pale green and grafted hydra which contained, respectively, 15% and 30% of the normal complement of algae lost material at a lower rate. Green hydra lost material at about half the rate of albinos and retained labeled material about twice as long. The relationship between rate of loss of labeled material by a group of hydra and its algal content is similar to that illustrated by the curves in Figure 4, where relatively few algae have nearly the same effect on the host as does a full complement of algae. The ability of the algae in grafted individuals to modify the rate of loss of material from the labeled albino body, despite the localization of algae in the unlabeled head, exemplifies a "replacement therapy" type of experiment, and suggests that the algae in this case might act by releasing something which diffuses through a distance.

That less material appeared in the medium of green and pale green hydra than in the medium of albinos implied that the algae either (1) directly affected the catabolic activities of the host cells, or (2) accumulated the labeled material after it was released by the animal cells. This was investigated in preliminary experiments in which sulfur-labeled hydra were starved for five days and then homogenized

by ultrasonic vibration. By gentle centrifugation most of the algae were separated from the bulk of the animal tissues, but mutual contamination could not be avoided. In two trials the resulting algal pellet contained 3.8% and 6.8% of the total radioactivity in the entire homogenate, tentatively indicating that the algae did not accumulate the isotope but depressed the rate of protein catabolism of the host.

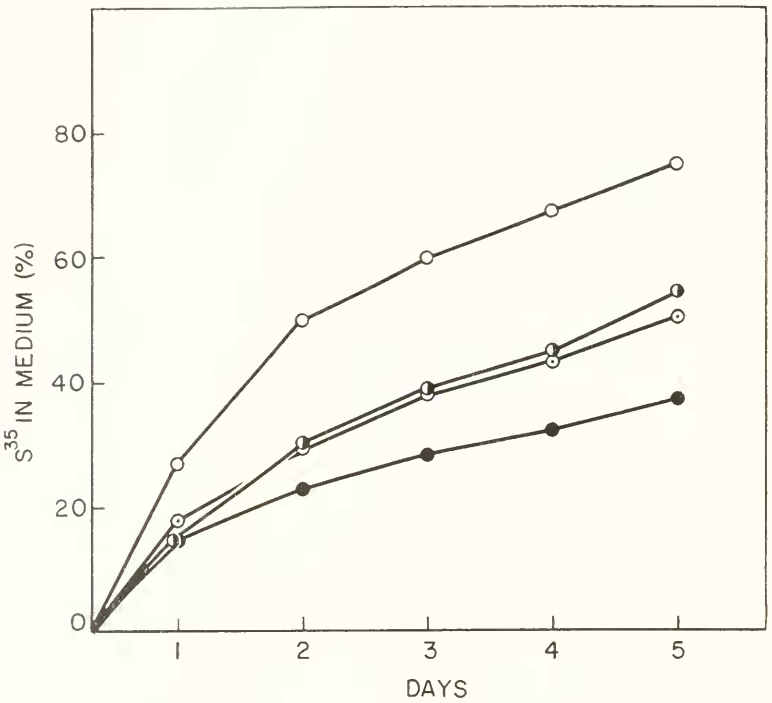

FIGURE 5. Rate of loss of S^{35}-labeled material by green (●), grafted (◑), pale green (⊙), and albino (○) *C. viridissima* containing, respectively, 100%, 30%, 20% and 0% photosynthetically-active plant material.

DISCUSSION

The results of this study lead to the conclusion that symbiotic algae favorably influence the growth, reproduction, survival, and protein turn-over of *C. viridissima*. The growth rate of green *C. viridissima* on a limited food supply was consistently greater than that of aposymbiotic controls (Fig. 2; Table I). This difference was not the result of a proportionally larger food intake by green hydra (Fig. 3), but of some intrinsic factor associated with the presence of algae, since even during starvation green hydra produced more buds, survived longer, resisted disintegration and displayed a lower turnover of sulfur-labeled protein compared to aposymbiotic controls (Tables II, III). These results lend quantitative support to the observations of Goetsch (1924), who noted that (1) well-fed albino hydra grew as well as well-fed green individuals, either in light or in darkness, and (2) when starved in the light, green hydra survived for nearly twice as long

as aposymbiotic controls. Partially "infected" hydra survived at least as long as any of the aposymbiotic controls and appeared "less depressed." Goetsch concluded that the algae were not essential when food was abundant but probably played some role in augmenting survival when certain stresses, *e.g.,* starvation, were imposed on the host. Karakashian (1963) demonstrated a positive influence of algae on growth of the ciliate protozoan, *Paramecium bursaria,* when bacterial food was present in low concentration and when cultures were starved but illuminated. A positive correlation of mean numbers of algae per paramecium with growth rate and survival time was also observed. Our results thus parallel these in several respects.

The adaptive value of symbiosis with algae is particularly evident from the behavior of albinos in these experiments. Their low budding rate and tendency to disintegrate early during starvation, and poor growth on a limited food supply would be disadvantageous for survival in an environment where limited food and periods of starvation are frequently encountered (Welch and Loomis, 1924). These observations perhaps explain why aposymbiotic adult *C. viridissima* have not yet been found in natural waters, although algae-free eggs are often produced by at least one strain of green and albino *C. viridissima* (unpublished observations).

Possible mechanisms by which the algae influence growth of the host

1. *Gas exchange and waste uptake*

Geddes (1882) suggested that symbiotic algae augment the well-being of their animal hosts in several ways, including (1) by taking up carbon dioxide and producing oxygen during photosynthesis, thus facilitating host respiration, and (2) by taking up host excretory wastes, such as ammonia, thereby creating a less toxic micro-environmental milieu for the animal. These interactions undoubtedly take place to some extent in most associations but as yet there is little direct evidence that any of them are essential to the animal (see Droop, 1963). In fact, they appear to be non-essential for *C. viridissima* since individuals without algae grow at k_{max} as long as they are well fed. Similarly, well-fed green and albino *C. viridissima* grow at nearly identical rates in darkness where photosynthetic gas exchange is again ruled out as an augmenting factor (Goetsch, 1924; our unpublished observations).

2. *Utilization of algal metabolic products*

As suggested by Geddes (1882) and others (Keeble, 1908; Boschma, 1925; Gohar, 1940, 1948) a host could benefit by digesting its symbiotic algae or utilizing their extracellular products. On the basis of the observations in this study, little can be said regarding digestion of algae by *C. viridissima.* Since there is no apparent decrease in number of algae after two to three weeks' starvation, and since 10–20% of the normal flora can sustain the starving host, digestion of algae seems unlikely but is not ruled out. As noted by Yonge (1944) symbiotic algae probably resist digestion since the majority are found in animals which display intracellular digestion.

However, there is evidence that *C. viridissima* utilizes products of algal metabolism. Experiments with $C^{14}O_2$ show that 10–20% of the labeled carbon fixed by the algae is transferred to the animal where some is incorporated into major chemical fractions (nucleic acids, proteins, etc.). The specific activity of C^{14} in algae-free green hydra tissues in these experiments was 50–100 times greater than that in albino control tissues where some carbon was assimilated solely by heterotrophic fixation (Lenhoff and Zimmerman, 1959; Muscatine and Lenhoff, 1963). Similar transfers take place in other coelenterate-algae associations (Muscatine and Hand, 1959; Goreau and Goreau, 1960) and are implied to occur in others (Sargent and Austin, 1949, 1954; Odum and Odum, 1955; Burkholder and Burkholder, 1960).

The results of this study and demonstration of the utilization of products of algal metabolism by host cells lend support to the conclusion that the algae in *C. viridissima* augment growth of the host by nutritional supplementation. Support for this view comes also from the observation that adequate food can replace the need for symbiotic algae (Goetsch, 1924; Fig. 2, this paper). A similar observation was reported by Parker (1926) and Karakashian (1963) for *P. bursaria.* The inability of albino *C. viridissima* to withstand starvation and the tendency to disintegrate undoubtedly reflects a loss of function by this species. Neither green *C. viridissima* nor the non-symbiotic species, *H. littoralis,* show this reaction to starvation, which could be symptomatic of a nutrient deficiency, as a result of a metabolic lesion. The growth lags and slow responses of albinos to intermittent feeding (Fig. 2) may represent the time needed to accumulate essential nutrients from the limited food supply. In contrast, green hydra did not exhibit extended growth lags or delayed responses to intermittent feeding. Auxiliary metabolites received from the algae probably offset any deficiency, though only temporarily, since, as shown in starvation experiments (Table II), the algae cannot sustain the animal indefinitely without some exogenous food. Information on carbon turnover rates by the algae, their extracellular products, the growth requirements of the host, and the peculiarities of the metabolism of algae-free individuals should bring to light the details of mechanisms of host-symbiont interaction in this association.

Part of this investigation was carried out at the Laboratories of Biochemistry, Howard Hughes Medical Institute, Miami, Florida during the tenure of a Postdoctoral Fellowship from the Division of General Medical Sciences, United States Public Health Service (1963) to Leonard Muscatine, and an Investigator Award of the Howard Hughes Medical Institute to Howard M. Lenhoff. We thank Mr. J. Bovaird, Mr. Alfredo Lopez, and Mr. Enrique Nagid for technical assistance.

Note Added in Proof

Slobodkin (1964) has recently demonstrated that the "ecological efficiency" (yield energy/food energy) of experimental populations of *C. viridissima* is about four times higher than that of *Hydra littoralis* (a non-symbiotic species), but only in populations grown in the light. The implication is that photosynthetic carbon is available to *C. viridissima* for energy. Slobodkin, L. B., 1964. Experimental populations of Hydrida. *J. Ecol. (Suppl.)*, **52**: 131–148.

Summary

1. When fed daily on *Artemia* nauplii, green and albino *C. viridissima* grew at nearly identical logarithmic rates. With limited food, growth of green hydra always exceeded that of albinos. This difference was not the result of a quantitative difference in food intake.

2. Green hydra survived starvation for about four weeks, gradually diminishing in size. Albinos survived only 10–12 days, succumbing to starvation by relatively sudden disintegration.

3. The relationship between survival ability and algal content was non-linear. Animals with 20% of the normal flora survived nearly as well as those with a full complement of algae.

4. Turnover rate of sulfur-labeled protein during starvation showed the relationship albino > pale green > green, among the groups tested. The presence of symbiotic algae appears to depress the rate of protein catabolism.

5. It is concluded that symbiotic algae augment growth, budding, and survival of *C. viridissima* (Carolina strain 1960) by a mechanism which does not appear to involve gas exchange or waste removal by the algae.

6. Evidence is presented in support of the hypothesis that algal metabolic products augment growth and survival of *C. viridissima*.

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